The Specificity of some Pig and Human Pepsins towards Synthetic Peptide Substrates

By ANDREW P. RYLE and CATHERINE A. AUFFRET*
*Department of Biochemistry, University of Edinburgh Medical School,
Teviot Place, Edinburgh EH8 9AG, Scotland, U.K.*

1. The peptidase activities of pig pepsins A and C and human pepsin and gastricsin were compared. 2. The peptides studied had the general formula A-Leu-Val-His-B. Hydrolysis at 37°C and pH 2.07 occurred at the amino side of the leucine residue for all the enzymes and all the peptides. 3. When A was Ac-Ala the peptides were hydrolysed under these conditions slowly by pig pepsin C only. 4. Pig pepsin A and human pepsin were unable to hydrolyse the tyrosine-containing peptides under the conditions tested. Gastricsin (human pepsin C) had about one-third of the activity of pig pepsin C with these substrates. 5. The increase in the rate of hydrolysis caused by the extension of the chain by a single alanine residue was most marked for pig pepsin A and human pepsin.

The presence of more than one pepsin in many animals has been known for some years. The minor pepsins B, C and D have been isolated from crude commercial pig pepsin and the corresponding zymogens have been isolated from the gastric mucosa of pigs (Ryle, 1970). Tang *et al.* (1959) isolated gastricsin, which is similar to pepsin C, from human gastric juice, and other components from human gastric juice have been described but not characterized in detail (e.g. Whitecross *et al.*, 1974). The present paper compares the activities of pepsins A and C from the pig and pepsin and gastricsin from man towards some small synthetic substrates. In this paper, following Tang (1970), the two human enzymes studied will be called pepsin and gastricsin, since they were isolated by his methods. A more detailed study of the hydrolysis of the substrates by pig pepsin C is reported in the preceding paper (Auffret & Ryle, 1979).

Materials and Methods

The pepsin C preparation was that described by Auffret & Ryle (1979). Pepsin A was obtained as a by-product of the preparation of pepsin D from crude pig pepsin (Lee & Ryle, 1967). Gastricsin and human pepsin were prepared by the method of Tang (1970) except that, since we consistently find that the pepsin is eluted by the pH 3.8 buffer used by Tang (1970) to elute inactive proteins, we used 0.2M-citrate buffer, pH 3.4, to wash the column before applying the pH 3.8 buffer. We find that the pepsin is eluted at pH 3.7 and the gastricsin at pH 4.23. The gastricsin was re-

* Present address: M.R.C. Biochemical Parasitology Unit, Molteno Institute, Downing Street, Cambridge CB2 3EE, U.K.

chromatographed on DEAE-cellulose with 0–0.2M-NaCl in 0.1M-acetate buffer, pH 4.0, in a 500ml constant-volume gradient maker. It emerged at 0.15M-NaCl (calculated). The specific activities of the enzymes were 35.7 and 13 m$[P.U.]^{Hb} \cdot ml^{-1} \cdot A_{280}^{-1}$ for porcine pepsins C and A respectively, measured by the assay of and using the units $[P.U.]^{Hb}$ of Ryle (1970). Specific activities of the human enzymes were measured with the haemoglobin assay of Tang (1970) and were 85 $\Delta A_{280} \cdot ml^{-1} \cdot A_{280}^{-1}$ for gastricsin and 39.5 $\Delta A_{280} \cdot ml^{-1} \cdot A_{280}^{-1}$ for pepsin.

Concentrations of the enzymes were measured by reading the absorbance of a solution of the enzyme in buffer or water at 280nm. For the pig pepsins and human pepsin A a solution of freeze-dried enzyme (1 mg/ml) gave an absorbance of 1.3 units. A similar value was assumed for gastricsin, which was kept frozen in solution after the final purification. Molecular weights of 36000 for pepsin C (Ryle & Hamilton, 1966) and 34500 for pepsin A, and the values quoted by Tang (1970) of 31400 for gastricsin and 34000 for human pepsin, were used.

Details of the preparations of the substrates, the use of the Technicon Auto-Analyzer and the handling of the data are given in the preceding paper (Auffret & Ryle, 1979). Ac-Ala-Phe-Leu-Val-His-NH₂ was not very soluble in the buffer and therefore a single stock solution was prepared, which was nominally 0.5mM. Insoluble material was removed by centrifugation, and an amino acid analysis was performed to establish the concentration of substrate in solution.

The hydrolysis of the peptides by pepsin C has already been described (Auffret & Ryle, 1979). The susceptibility of each of the substrates to hydrolysis by each of the other enzymes at pH 2.07 and 37°C was

tested, an enzyme concentration in the incubation mixture of 2–3 μg/ml being used, which gave a rapid rate of hydrolysis by pepsin C.

Results and Discussion

Paper electrophoresis at pH 3.6 (30 V/cm; 75 min) showed that substrate hydrolysis occurred (if at all) exclusively at the amino-terminal side of the leucine residue. Neither Ac-Ala-Leu-Val-His-OMe nor the corresponding amide was hydrolysed by gastricsin or the pepsins under the conditions used, and no further studies were made with these substrates. Results for the other substrates are shown in Table 1.

Gastricsin, the human equivalent to pepsin C, showed specificity similar to that of the pig enzyme, but was found to be very much less active towards the peptide substrates, although its activity

towards haemoglobin as substrate was comparable with that of pepsin C by the assays of both Ryle (1970) and Tang (1970).

The phenylalanine peptides were hydrolysed by all of the enzymes, but most rapidly by pepsin C, and for each the inclusion of the extra alanine residue increased the value of $k_{cat.}/K_m$. This effect is less marked for gastricsin than for the other enzymes: its $k_{cat.}$ is decreased. The general lowering of K_m, implying tighter binding of the substrate, with this increase in substrate size agrees with earlier observations for pepsin A, which have led to proposals for secondary binding sites on the enzyme (Fruton, 1970, 1976).

The tyrosine peptides were not hydrolysed by pig pepsin A or by human pepsin when tested with 0.5 mM-substrate, 2 μg of enzyme/ml at pH 2. Very slow hydrolysis of Ac-Tyr-Leu-Val-His-NH$_2$ by human pepsin was detected when a 10-fold greater enzyme concentration was used.

Table 1. *Kinetic parameters for the hydrolysis of peptides by pig and human pepsins at 37°C*
a, Ratio of individual $k_{cat.}$ and K_m. *b*, Values from initial slope of plot of v versus [S].

Substrate and enzyme	pH	Range of [S] (mM)	K_m (mM)*	$k_{cat.}$ (s^{-1})*	$k_{cat.}/K_m$ (s$^{-1} \cdot$ mM^{-1})	
					a	*b*
Ac-Phe-Leu-Val-His-NH$_2$						
Pepsin C (pig)	2.1	0.5–1.4	1.84† (1.07–2.52)	6.12† (4.34–7.79)	3.32†	2.86†
Pepsin A (pig)	2.1	0.25–1.25	1.51 (1.28–1.53)	0.37 (0.33–0.38)	0.25	0.21
Gastricsin (man)	2.1	0.25–1.25	2.76 (2.67–3.81)	0.60 (0.58–0.79)	0.22	0.21
Pepsin (man)	2.1	0.25–1.25	3.24 (2.00–4.35)	0.28 (0.19–0.35)	0.086	0.08
Ac-Ala-Phe-Leu-Val-His-NH$_2$						
Pepsin C (pig)	2.1	0.075–0.375	0.70† (0.48–1.39)	7.01† (5.48–11.86)	10.1†	9.11†
Pepsin A (pig)	2.1	0.038–0.375	0.87 (0.81–1.04)	5.80 (5.42–6.70)	6.6	6.03
Gastricsin (man)	2.1	0.038–0.375	0.51 (0.36–0.73)	0.30 (0.22–0.40)	0.58	0.53
Pepsin (man)	2.1	0.038–0.375	0.91 (0.58–8.9)	3.4 (2.3–26)	3.7	3.49
Ac-Tyr-Leu-Val-His-OMe						
Pepsin C (pig)	2.1	0.025–0.15	0.28† (0.24–0.31)	1.06† (0.99–1.22)	3.81†	3.45†
	3.0	0.025–0.1				4.1‡
Gastricsin (man)	2.1	0.025–0.2				1.18§
	3.0	0.025–1.0				0.83 ± 0.012 (s.d.)‖
Ac-Tyr-Leu-Val-His-NH$_2$						
Pepsin C (pig)	2.1	0.1–0.5	0.82† (0.49–1.22)	1.07† (0.69–1.37)	1.31†	1.16†
Gastricsin (man)	2.1	0.1–0.5				0.288 ± 0.004 (s.d.)‖

* Values in parentheses are 95% confidence limits.
† Data from Auffret & Ryle (1979).
‡ Interpolated from pH-dependence of $k_{cat.}/K_m$.
§ Initial slope of upward-curving plot of v versus [S].
‖ From linear regression of plot of v versus [S].

The tyrosine peptides with pepsin C and gastricsin gave plots of v versus [S] that were either linear or showed signs of activation by substrate. No attempt was made to determine separate values of $k_{cat.}$ or K_m, but values of $k_{cat.}/K_m$ were determined directly. (The value for pepsin C with Ac-Tyr-Leu-Val-His-OMe at pH 3.0 shown in Table 1 is a value interpolated from a plot of this parameter against pH.) It is not possible to give formal estimates of the errors for the values obtained from curved plots; they should be of the same order as those obtained from linear ones.

Pepsin C was again found to be more active than gastricsin. In general the pig enzymes seem to show greater activity in the hydrolysis of small synthetic substrates, but it is not possible to say whether there is any biological significance in this observation.

The low ability of pig pepsin A and human pepsin to hydrolyse the peptides at the tyrosine–leucine bonds is in agreement with work published by Fruton (1970, 1976) for similar peptides. There must be some differences in the active-site regions of pig pepsin C and pepsin A such that pepsin C can accommodate the phenolic hydroxy group, but pepsin A cannot. This may be an effect of the size of the side chain, or perhaps pepsin C contains a residue which stabilizes the presence of a hydroxy group, by hydrogen-bonding, whereas pepsin A does not. This may be in accord with the finding of Fruton (1970) that masking the phenolic hydroxy group with a methyl group partly relieves the inhibitory effect of the tyrosine residue. The ability of pepsin A to hydrolyse tyrosyl bonds in proteins and polypeptides (Tang, 1963;

Hill, 1965) perhaps reflects the importance of secondary binding sites in these cases.

Tang (1970) has described a method of differential assay of pepsin and gastricsin in samples of human gastric juice, with Ac-Phe-Tyr(I$_2$) and haemoglobin as substrates. It seems likely that Ac-Tyr-Leu-Val-His-NH$_2$ could usefully replace haemoglobin in such a scheme. The validity of such a method would depend on the activities with the peptide substrates of the other minor acid proteinases detectable in human gastric juice, and these have not yet been tested.

References

Auffret, C. A. & Ryle, A. P. (1979) *Biochem. J.* **179**, 239–246

Fruton, J. S. (1970) *Adv. Enzymol.* **33**, 401–443

Fruton, J. S. (1976) *Adv. Enzymol.* **44**, 1–36

Hill, R. L. (1965) *Adv. Protein Chem.* **20**, 37–107

Lee, D. & Ryle, A. P. (1967) *Biochem. J.* **104**, 742–748

Ryle, A. P. (1970) *Methods Enzymol.* **19**, 316–336

Ryle, A. P. & Hamilton, M. P. (1966) *Biochem. J.* **101**, 176–183

Tang, J. (1963) *Nature (London)* **199**, 1094–1095

Tang, J. (1970) *Methods Enzymol.* **19**, 406–421

Tang, J., Wolf, S., Caputto, R. & Trucco, R. E. (1959) *J. Biol. Chem.* **234**, 1174–1178

Whitecross, D. P., Clark, A. D. & Piper, D. W. (1974) *Scand. J. Gastroenterol.* **9**, 711–717